Identification in Vitro of a Post-Translational Regulatory Site in the Hinge 1 Region of Arabidopsis Nitrate Reductase

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Nitrate reductase (NR) is rapidly inactivated by phosphorylation of serine residues in response to loss of light or reduction in CO_2 levels. To identify sites within NR protein that play a role in this post-translational regulation, a heterologous expression system and an in vitro inactivation assay for Arabidopsis NR were developed. Protein extracts containing NR kinases and inhibitor proteins were prepared from an NR-defective mutant that had lesions in both the *NIA1* and *NIA2* NR genes of Arabidopsis. Active NR protein was produced in a *Pichia pastoris* expression system. Incubation of these two preparations resulted in a Mg-ATP–dependent inactivation of NR that was reversed with EDTA. Mutant forms of NR were constructed, produced in *P. pastoris*, and tested in the in vitro inactivation assay. Six conserved serine residues in the hinge 1 region of NR, which separates the molybdenum cofactor and heme domains, were specifically targeted for mutagenesis because they are located in a potential regulatory region identified as a target for NR kinases in spinach. A change in Ser-534 to aspartate was found to block NR inactivation; changes in the other five serines had no effect. The aspartate that replaced Ser-534 did not appear to mimic a phosphorylated serine but simply prevented the NR from being inactivated. These results identify Ser-534, located in the hinge 1 of NR and conserved among higher plant NRs, as an essential site for post-translational regulation in vitro.

INTRODUCTION

A primary mechanism for acquiring nitrogen from the environment is to take up and assimilate nitrate. Nitrate is actively transported into the cell and then reduced to ammonia in two steps before being incorporated into glutamine. This simple pathway relies on elaborate regulatory networks that respond to a wide range of environmental and internal signals, including nitrate, light and CO_2 levels, circadian rhythms, nitrogen and carbon metabolites, and phytohormones. Such complex regulation is needed to coordinate nitrate assimilation with other key metabolic processes such as carbon metabolism and photosynthesis (reviewed in Hoff et al., 1994; S.C. Huber et al., 1994; Huppe and Turpin, 1994; Crawford, 1995).

The first committed step in the nitrate assimilation pathway is catalyzed by nitrate reductase (NR), a cytosolic enzyme that produces nitrite. Nitrite reductase catalyzes the subsequent step producing ammonia in the plastid. The regulation of NR involves both transcriptional and post-transcriptional mechanisms (reviewed in Crawford and Arst, 1993; Hoff et al., 1994; S.C. Huber et al., 1994; Kaiser and Huber, 1994b; Crawford, 1995). These mechanisms regulate both the amount and

activity of NR protein in the cytosol. Post-translational mechanisms reversibly modulate NR activity within minutes and permit quick responses to changes in key metabolites or reductant levels in the cell. For example, NR activity is tightly coordinated with nitrite reductase activity to prevent the accumulation of nitrite, which is toxic to plants (Riens and Heldt, 1992; Vaucheret et al., 1992; Duncanson et al., 1993).

To control NR activity, plants rapidly inactivate NR in response to several signals: the loss of light, a decrease in CO_2 levels, or an increase in cytosolic pH (Kaiser and Brendle-Behnisch, 1991, 1995; J.L. Huber et al., 1992, 1994; MacKintosh, 1992; Riens and Heldt, 1992; de Cires et al., 1993; Lillo, 1994a; Kojima et al., 1995). Inactivation correlates with an increased phosphorylation of NR on specific serine residues and is dependent on millimolar concentrations of magnesium or calcium (Kaiser and Brendle-Behnisch, 1991; J.L. Huber et al., 1992, 1994). In vitro, NR can be inactivated in desalted protein extracts in the presence of Mg-ATP (Kaiser and Spill, 1991; Huber et al., 1992; MacKintosh, 1992; Kaiser and Huber, 1994a; Lillo, 1994b; Glaab and Kaiser, 1995; Kojima et al., 1995; Nussaume et al., 1995). Purification of these protein extracts has revealed at least two calcium-dependent kinases that phosphorylate NR and an inhibitor protein (called NiP or IP) that inactivates NR stoichiometrically in the presence of magnesium (Spill and

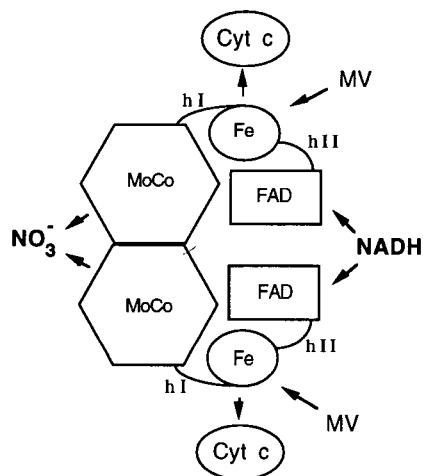[1] To whom correspondence should be addressed.

Kaiser, 1994; Bachmann et al., 1995; Glaab and Kaiser, 1995; MacKintosh et al., 1995; McMichael et al., 1995). Thus, NR inhibition requires phosphorylation of specific serine residue(s) and presumably binding of NiP in the presence of physiological concentrations of magnesium. Reactivation of NR is induced by dephosphorylation by protein phosphatase 2A, which can be inhibited both in vivo and in vitro with the inhibitors okadaic acid and microcystin (Huber et al., 1992; MacKintosh, 1992; Kaiser and Huber, 1994a, 1994b; Kojima et al., 1995; MacKintosh et al., 1995).

One of the critical unanswered questions in this system is which residues in NR are the targets for the kinases and NiP. NR has three functional domains, each associated with a reactive center (flavin adenine dinucleotide [FAD], heme, or molybdenum-pterin cofactor [MoCo]) that shuttles electrons from NAD(P)H to nitrate (Figure 1). Electrons are transferred first from the NAD(P)H to FAD, then to the heme group, and lastly to MoCo, which reduces nitrate. Alternative electron donors or acceptors can be used in vitro that require only one or two of the functional domains of NR and give partial reactions. If the individual domains of inactivated NR are assayed using the partial reactions, one finds that electron transfer from the heme to nitrate is impaired. Inactivated NR cannot reduce nitrate with methyl viologen but can support cytochrome c reduction by NADH (Huber et al., 1992; MacKintosh et al., 1995). Consistent with these biochemical data, a mutation has been found in the MoCo domain of the Arabidopsis NR (a glycine-to-aspartic acid mutation at position 308) that dramat-

Figure 1. Diagram of the Functional Domains of NR and Partial Activities.

Functional domains and the two hinge regions are as defined by Crawford et al. (1988) and Campbell and Kinghorn (1990). MV transfers electrons to the heme-Fe domain, which then reduces MoCo and nitrate. Cytrochome c (Cyt c) receives electrons from NADH via the FAD and heme domains. hI and hII, hinge 1 and hinge 2; Fe, heme iron; MV, reduced methyl viologen.

ically reduces the level of NR phosphorylation (LaBrie and Crawford, 1994). In addition, a 56–amino acid deletion in the N-terminal leader region of Nicotiana plumbaginifolia NR (just preceding the MoCo domain) has been shown to block the ATP-dependent inactivation of NR in vitro and the post-transcriptional regulation by light in vivo (Nussaume et al., 1995). Thus, phosphorylation and post-translational regulation of NR appear to involve the MoCo domain or residues closely associated with it.

Additional data provide clues about which residues are phosphorylated in NR. Phosphoamino acid analysis shows that only serine is phosphorylated (J.L. Huber et al., 1992, 1994; LaBrie and Crawford, 1994). Phosphopeptide analysis has revealed a more complicated picture in which one or more serines are phosphorylated when NR is inactivated in addition to other serines that are constitutively phosphorylated (J.L. Huber et al., 1992, 1994; LaBrie and Crawford, 1994). However, recent results indicate that a single serine in the hinge 1 region of spinach NR is a regulatory phosphorylation site (Bachmann et al., 1996). Partially purified preparations of spinach NR kinases efficiently phosphorylate fragments or oligopeptides (as short as 18 amino acids) of NR only if they include Ser-543 and several conserved residues surrounding this site. Based on sequence analysis, Ser-543 makes an excellent calcium-dependent protein kinase (CDPK) phosphorylation site, which is consistent with biochemical data showing that NR kinases are calcium dependent and are most likely CDPKs (Bachmann et al., 1995, 1996; McMichael et al., 1995). These results are especially interesting because hinge 1 has not been known to play any role other than connecting the MoCo and heme domains. If one could determine the effect of mutating this region of NR, the role of Ser-543 as a regulatory site could be verified and the function, if any, of other hinge 1 residues could be determined.

The study of NR structure and regulation has been impeded by the lack of a workable heterologous expression system to produce wild-type and mutant forms of holo-NR for in vitro analysis. Partial success has been reported for tobacco NR, which was expressed in Saccharomyces cerevisiae, producing a partially functional NR lacking MoCo (Truong et al., 1991). In this study, we report the successful production of active holo-NR (i.e., containing FAD, heme, and MoCo), using the yeast Pichia pastoris. In addition, an in vitro inactivation assay was developed to test NR produced in the expression system. For the assay, we took advantage of an Arabidopsis mutant with almost no endogenous NR activity due to mutations in both NR structural genes, NIA1 and NIA2 (Wilkinson and Crawford, 1993). Extracts from this mutant provide a source of NR kinase and NiP that can be incubated with exogenous NR. Because Ser-543 in spinach is conserved among almost all known higher plant NRs, the corresponding serine in Arabidopsis NR (residue 534) was mutated to determine whether it is required for in vitro inactivation, as indicated by the biochemical work in spinach. In addition, other conserved serines in hinge 1 were examined. This article describes the results of these experiments.

Table 1. Comparision of NR and MoCo Activity

Sources	NR Activity[a]	MoCo Activity[a]
Arabidopsis	0.020	0.016
P. pastoris (pHILD2 vector)	0[b]	0.015
P. pastoris (pHILD2-wtNR)	0.08[b]	ND[c]
S. cerevisiae (INVS1)	0	0

[a] NR and MoCo activity are given in units per milligram of total protein as described in Methods.
[b] Transformants were grown for 24 hr in induction medium.
[c] ND, Not determined.

RESULTS

Wild-Type and Mutant Forms of Arabidopsis NR Can Be Produced in P. pastoris

Our first goal was to find an expression system to produce NR. It had been reported that NR could be produced in *S. cerevisiae*, but the NR lacked MoCo (Truong et al., 1991). *S. cerevisiae* extracts were tested for MoCo activity by using the *Neurospora crassa nit-1* reconstitution assay (LaBrie et al., 1992). No activity was found (Table 1). We then tested the methylotrophic yeast *P. pastoris* and found it had MoCo activity (0.015 units per mg) at levels comparable with those measured in Arabidopsis extracts (Table 1). Because *P. pastoris* has been used extensively for expressing heterologous proteins (Cregg et al., 1993), we asked whether it could produce functional NR from

a full-length NR cDNA clone from Arabidopsis. The *P. pastoris* alcohol oxidase promoter, which is inducible by methanol and produces alcohol oxidase up to 30% of total soluble protein, was used to drive the expression of NR cDNA. As seen in Table 1, *P. pastoris* transformed with the NR expression plasmid pHILD2-wtNR produced NR activity when induced with methanol, whereas *P. pastoris* containing only a control expression plasmid (pHILD2) had no such activity. The NR activity detected in *P. pastoris* was inhibited by anti–Arabidopsis NR antibody (data not shown).

Given that holo-NR can be expressed in *P. pastoris*, potential regulatory sites in NR could now be mutagenized and tested. Conserved serines in the hinge 1 region separating the MoCo and heme domains were mutated. This region is not thought to be an integral part of the functional domains of NR because its sequence is not similar to other MoCo- and cytochrome *b*–containing enzymes, such as sulfite oxidase (Campbell and Kinghorn, 1990). Yet, there are many highly conserved residues in this region among higher plants (Figure 2), including several that are potential substrates for CDPK.

Seven serines in or adjacent to hinge 1 were mutated, as highlighted in Figure 2. These serines were changed to alanine or aspartate and designated S509A, S509D, S522A, S522D, S532A, S532D, S534A, S534D, S546A, S546D, S548A, S548D, S555A, and S555D based on the numbering system previously reported (Crawford et al., 1988). Ser-534 corresponds to Ser-543 in spinach NR, the latter being a target for spinach NR kinases (Bachmann et al., 1996). After introducing these mutant cDNAs (subcloned into a *P. pastoris* expression plasmid) into the *P. pastoris* strain GS115 (see Methods), all expressed functional NR except S532A, S532D, S534A, S534D, and S555A. Subsequently, it was found that
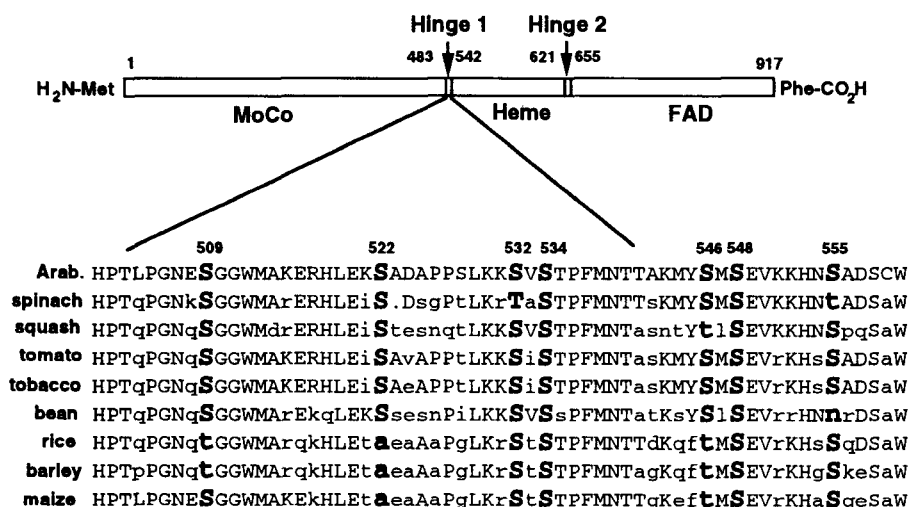
Figure 2. Amino Acid Sequence Alignment of the Hinge 1 Region and Mutated Serines.

The top line is a diagram of NR. The sequence data below were obtained from GenBank (accession number J03240). Boldface S's are the serines that were mutated to alanine (A) and aspartate (D) in the Arabidopsis NR gene. Arab., Arabidopsis *NIA2* gene.
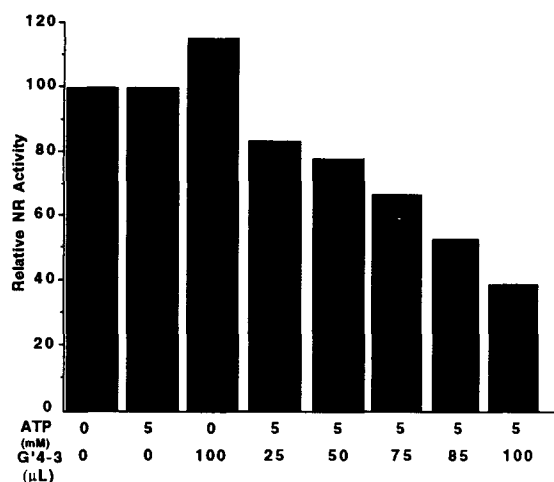
Figure 3. In Vitro Inactivation of Wild-Type NR Expressed in *P. pastoris*.

P. pastoris-produced NR was incubated with different amounts of ATP and desalted extracts from the NR-defective mutant G'4-3 for 20 min and then assayed for NR activity. Protein extractions, in vitro inactivation, and NR assays were as described in Methods. The specific activity for the wild-type NR was 0.015 units per mg of protein, and the protein concentration for G'4-3 was 4 mg/mL. The amount of G'4-3 extract added and the concentration of ATP in the in vitro inactivation assays are given below the histogram.

the S534D cDNA expressed functional NR enzyme in the proteinase-defective strain of *P. pastoris* SMD1168 (White et al., 1994). We do not know why the other four did not produce active NR in *P. pastoris* but presume that they were hypersensitive to proteases due to their specific mutations. Thus, functional NR proteins mutated at each one of six possible sites in hinge 1 were produced.

## *P. pastoris*-Produced NR Can Be Inactivated in Vitro

Several studies have shown that NR in desalted plant extracts from spinach, *Brassica campestris*, and *N. plumbaginifolia* can be inactivated in vitro in the presence of Mg-ATP (Kaiser and Spill, 1991; Huber et al., 1992; MacKintosh, 1992; Kaiser and Huber, 1994a; Lillo, 1994b; Glaab and Kaiser, 1995; Kojima et al., 1995; Nussaume et al., 1995). This inhibition requires kinase activity and an NiP. We tested whether Arabidopsis NR made in *P. pastoris* could be inactivated in vitro by using protein extracts from Arabidopsis. An NR-defective, double mutant of Arabidopsis G'4-3 was used for this purpose (Wilkinson and Crawford, 1993). This mutant has only 0.5% of the wild-type level of NR activity due to a deletion of the major NR gene *NIA2* and an ethyl methanesulfonate–induced mutation in the minor NR gene *NIA1*. G'4-3 should contain a protein kinase(s) as well as the NR NiP. The addition of desalted G'4-3 extract to NR produced in *P. pastoris* had little effect on the total NR

activity if assayed immediately (data not shown), although a 20-min incubation with G'4-3 increased NR activity ∼10% (Figure 3). If Mg-ATP was included in the reaction, NR activity decreased substantially (Figure 3). The decrease was a function of the amount of G'4-3 extract added (Figure 3). These results are consistent with previous findings in which inactivation is a function of the NiP concentration, which acts stoichiometrically (Kaiser and Huber, 1994b; MacKintosh et al., 1995). Thus, the Mg-ATP–dependent inactivation of NR˙ could be reconstituted in vitro by using *P. pastoris*–produced NR and protein extract from G'4-3.

## Effect of Serine Mutations on in Vitro Inactivaton

Mutants that expressed functional NR were subjected to the in vitro inactivation assay described above. Mutant forms of NR were mixed with G'4-3 extract with or without Mg-ATP and then assayed for NR activity. Wild-type and mutant NRs with alterations at residues 509, 522, 546, 548, and 555 were all inhibited in the presence of Mg-ATP (Figure 4). However, S534D NR was not inhibited but instead showed a small but reproducible activation (Figure 4). These results demonstrate that a mutation at S534 blocks the Mg-ATP–dependent inactivation of NR in vitro.

We verified that the S534D NR is as active as wild-type NR before inactivation by immunoblot analysis (Figure 5). The specific activity of wild-type NR in *P. pastoris* extracts was three times higher than that of the mutant NR; however, the amount
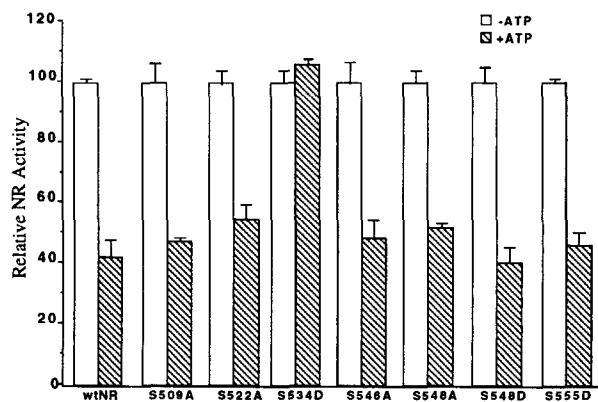
Figure 4. Inactivation of Mutant Forms of NR.

Twenty to 40 μL of NR protein expressed in *P. pastoris* was mixed with 150 μL of desalted G'4-3 extract and incubated at room temperature for 20 min, with (+) or without (−) 5 mM ATP, followed by an NR assay as described in Methods. The form of NR (wild type or mutant) added to the incubation is indicated below the histogram. Protein extraction and NR assay conditions are the same as in Figure 3. Specific activities of *P. pastoris*–expressed NR were 0.01 to 0.05 units per mg of protein. Protein concentration for G'4-3 extract was 4 mg/mL. The means and standard error represent three replicate experiments.
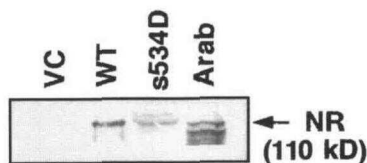
Figure 5. Immunoblot Analysis of the Wild-Type and S534D NR in *P. pastoris* Extracts.

Protein extracts were prepared from *P. pastoris* strains transformed with vector alone (VC), wild-type NR expression plasmid (WT), and S534D NR expression plasmid (s534D) and from wild-type Arabidopsis leaves (Arab). One hundred micrograms of total protein from each sample was electrophoresed on an 8% SDS–polyacrylamide gel, transferred to nitrocellulose, and incubated with antibodies raised against the Arabidopsis *NIA2* NR protein as described by Wilkinson and Crawford (1991), except that the membrane was blocked in 10% dry milk/Tris-saline and the first and secondary antibodies were suspended in 5% dry milk/Tris-saline. Specific activities for wild-type, S534D, and Arabidopsis NRs were 0.031, 0.010, and 0.015 units per mg of total protein, respectively, measured in the NR assay buffer described in Methods. The position of NR is calculated from Bio-Rad protein markers and indicated by the arrow. Quantitation of the 110-kD NR bands showed that S534D had 33.5% and Arabidopsis had 80% of the wild-type signal.

of wild-type NR protein is approximately three times higher than that of the mutant NR in the extracts. Thus, the S534D mutation does not appear to impair NR specific activity.

S534D Does Not Mimic a Phosphoserine Residue

In some systems, aspartate has been shown to mimic a phosphorylated serine. For example, in mammalian mitogen-activated protein (MAP) kinase kinase, replacing a key regulatory serine (Ser-222) with aspartate will constitutively activate the kinase as if it had a phosphoserine at position 222 (Pages et al., 1994). In sorghum phosphoenolpyruvate carboxylase, aspartate can functionally mimic a phosphoserine at position 8 and render the enzyme less sensitive to malate inhibition (Wang et al., 1992).

To determine how S534D behaves in NR, the magnesium sensitivity of the inactivation was examined. Phosphorylated NR normally requires millimolar concentrations of magnesium (or calcium) in the assay buffer to be inhibited (Kaiser and Brendle-Behnisch, 1991; Huber et al., 1992). Arabidopsis wild-type NR also displays a magnesium requirement (Figures 1 and 6). Incubation of wild-type NR with the G'4-3 extract with Mg-ATP inactivates NR, and as expected, omission of the extract or ATP prevents NR inactivation of wild-type NR (Figures 1 and 6). If the magnesium is removed with EDTA after NR is phosphorylated in the presence of the G'4-3 extract, wild-type NR is not inhibited (Figure 6); thus, the inactivation is reversed by EDTA treatment. If S534D behaved like a phosphoserine, the addition of G'4-3 and magnesium should drastically inhibit NR even in the absence of ATP. No such in-

hibition was seen (Figure 6). Furthermore, if S534D mimics a phosphoserine, removal of magnesium by EDTA from an NR plus G'4-3 mixture should increase the mutant NR activity two- to threefold, as was found for phosphorylated wild-type NR. Only a 10 to 15% increase was found when EDTA was added (Figure 6). Thus, S534D simply blocks the inactivation and does not mimic the phosphorylated state of the enzyme.

DISCUSSION

In this study, we report two advances in our study of metabolic regulation in plants. Specifically, we show that plant holo-NR can be produced in a yeast expression system. We also show that a highly conserved serine (residue 534) in the hinge 1 of Arabidopsis NR is required for the ATP- and Mg-dependent inactivation of NR in vitro. These results provide insights into the mechanisms that control biochemical pathways in plants and important tools for further elucidation of enzyme structure and regulation.

For some time, there has been a need for a system to express plant NR cDNAs for both structural and regulatory studies. Fragments of NR have been produced in bacteria and yeast (Hyde and Campbell, 1990; Campbell, 1992; Cannons et al., 1993; Cannons and Solomonson, 1994; Shiraishi and Campbell, 1995), and an apo-NR has been made in *S. cerevisiae* (Truong et al., 1991). It is not surprising that the yeast *S. cerevisiae* cannot make holo-NR because this yeast cannot
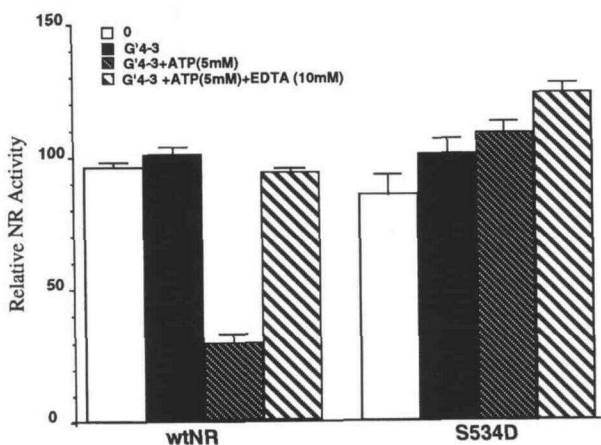
Figure 6. Magnesium Dependence of NR Inactivation.

The inactivation assay was as described in Figure 3. Specific activities for wild-type NR (wt NR) and S534D NR were 0.015 and 0.010 units per min per mg of protein, respectively. G'4-3 extract (180 mL at 5 mg/mL) was used in each treatment, except for the control (open bars). EDTA was added to a final concentration of 10 mM after a 20-min incubation at room temperature and immediately before the NR assay. Means and standard errors for three replicates are given.

use nitrate as a nitrogen source and lacks MoCo. An ideal species would be one that has MoCo, lacks endogenous NR, and has been developed as an expression system. *P. pastoris* is an excellent candidate because it cannot grow on nitrate as a nitrogen source (Hipkin, 1989) and it has been developed into a superb expression system (Cregg et al., 1993). We tested several *P. pastoris* strains and found that they have MoCo activity but no endogenous NR under the growth conditions tested. When an Arabidopsis NR cDNA driven by a *P. pastoris* promoter was introduced into these strains, the transformants expressed holo-NR. This breakthrough allowed us to make mutant forms of NR for our regulation studies. We have not optimized the production of NR in *P. pastoris*, but the levels of other proteins can exceed 1 gm/L (Cregg et al., 1993). Such a facile system should allow production of large quantities of NR for crystallography and biophysical characterization. Information from such studies will be important for elucidating the mechanisms that mediate substrate binding, electron transfer, and post-translational regulation.

Once the *P. pastoris* system was shown to work, Arabidopsis NR cDNA was subjected to mutagenesis to find regulatory target sites for the NR kinase(s). However, before we could examine mutant forms of NR, we needed an in vitro inactivation system. Extensive biochemical characterization of the in vitro inactivation of NR had been done for spinach but not for Arabidopsis. We adapted the spinach system for Arabidopsis and took advantage of the NR double mutant G'4-3. G'4-3 was critical for this study because it has so little endogenous NR activity that one can add large quantities of the extract to inhibit exogenous NR without introducing additional NR. The key finding was that *P. pastoris*-produced NR could be inactivated by extracts from G'4-3 when Mg-ATP was included. This system models the phosphorylation-dependent inactivation of NR observed in vivo and allowed us to test the effect of mutating specific serines in NR.

We focused our mutagenesis on specific serines in the hinge 1 region of NR for several reasons. This region has conserved residues that are sandwiched between but are not part of the heme and MoCo domains based on sequence comparisons. Such conservation suggests an important function that could include regulation. This region should be accessible to proteins, such as kinases, because it is readily digested by proteases that detach the MoCo domain from the heme domain (Solomonson and Barber, 1990). It has several serines (534, 546, and 555) that have the sequence "basic-X-X-serine" (where basic is K or R, and X is any amino acid), which is a substrate for an important class of protein kinases in plants, CDPKs (Roberts and Harmon, 1992). Most importantly, a serine in this region has been shown to be a target for NR kinases in spinach (Douglas et al., 1995; Bachmann et al., 1996).

When the conserved serines in hinge 1 were mutated, a surprising result was obtained. S534D completely blocked inactivation, whereas mutations at the other sites had little effect. This result indicates that S534 is directly involved in the regulation of NR. It is interesting that three amino acids near S534 are also highly conserved in plants and are critical for

recognition by mammalian Ca^{2+}/calmodulin–dependent protein kinase I and yeast SNF1 (sucrose nonfermenting) protein kinases (Lee et al., 1994; Dale et al., 1995). These residues include a hydrophobic amino acid at position +5 (five residues N-terminal) from the serine, a basic amino acid at +3, and a hydrophobic residue at -4. It is likely that these residues are a part of the substrate determinants for the NR plant kinases. Although S534 and adjacent amino acids are conserved among higher plant NRs, they are not conserved in algae or fungi, suggesting that this mechanism for phosphorylation-dependent inactivation of NR has evolved only in higher plants.

Our results add an important component to the model for NR inactivation and highlight the importance of hinge 1. Previously, hinge 1 was thought of as a spacer that held two domains together. It is clear that hinge 1 plays a vital regulatory role as well. The simplest model that best explains our current data is that NR is phosphorylated on Ser-534 and then binds the NR NiP, which in turn blocks the transport of electrons from the heme to nitrate. Further work is needed to verify this model.

Two primary mechanisms have been proposed in the literature for phosphorylation-mediated regulation of enzymes: direct perturbation of the enzyme's active site by the phosphoryl group or long-range effects induced by phosphorylation (Sprang et al., 1988; Dean and Koshland, 1990; Wang et al., 1992; Omkumar and Rodwell, 1994). The latter mechanism most likely applies to NR. That mutations far removed from the phosphorylation site can block NR inactivation or phosphorylation (LaBrie and Crawford, 1994; Nussaume et al., 1995) and that simple phosphorylation does not inactivate NR until an inhibitory protein interacts with it suggest that long-range conformational events are a part of the regulation. It is interesting that the aspartate group at position 534 did not mimic a phosphorylated serine. S534D NR was not inhibited but in fact showed a small but reproducible increase in activity when incubated with Arabidopsis extracts. Hinge 1 is clearly an important region for regulating NR activity. Additional experiments are needed to determine how hinge 1 mediates its control of NR activity.

METHODS

Site-Directed Mutagenesis of Nitrate Reductase cDNA

Standard molecular cloning techniques were used for DNA manipulation (Sambrook et al., 1989). Site-directed mutagenesis was performed as described by Higuchi (1990). The template for polymerase chain reaction was a 1.6-kb BamHI fragment (from nucleotide position 898 to 2509) from the *NIA2* nitrate reductase (NR) cDNA clone pAtc46 of Arabidopsis (Wilkinson and Crawford, 1991). Oligonucleotides used as primers to generate the mutations are shown in Figure 7. Polymerase chain reaction products were gel purified and then digested with AccI and HincII to produce a 1.2-kb (1098 to 2348) fragment. This fragment was then subcloned back into pAtc46 to replace the wild-type sequence. Finally, the modified region was sequenced and checked for the expected mutation and other possible errors.

S509A
5'-CCTGGTAATGAA<u>GCT</u>GGTGGATGGATG-3'
S509D
5'-CCTGGTAATGAA<u>GAT</u>GGTGGATGGATG-3'
S522A
5'-CACCTCGAAAAA<u>GC</u>GGCTGACGCGCCT-3'
S522D
5'-CACCTCGAAAAA<u>GAC</u>GCTGACGCGCCT-3'
S532A
5'-AGTCTAAAGAAG<u>GCT</u>GTCTCGACGCCG-3'
S532D
5'-AGTCTAAAGAAG<u>GAT</u>GTCTCGACGCCG-3'
S534A
5'-AAGAAGTCTGTC<u>GCG</u>ACGCCGTTTATG-3'
S534D
5'-AAGAAGTCTGTC<u>GAC</u>ACGCCGTTTATG-3'
S546A
5'-GCGAAGATGTAC<u>GCG</u>ATGTCCGAGGTC-3'
S546D
5'-GCGAAGATGTAC<u>GAC</u>ATGTCCGAGGTC-3'
S548A
5'-ATGTACTCGATG<u>GCC</u>GAGGTCAAGAAG-3'
S548D
5'-ATGTACTCGATG<u>GAC</u>GAGGTCAAGAAG-3'
S555A
5'-AAGAAGCATAAT<u>GCG</u>GCTGACTCTTGC-3'
S555D
5'-AAGAAGCATAAT<u>GAC</u>GCTGACTCTTGC-3'

Figure 7. Oligonucleotides Used for Mutagenesis.

The oligonucleotides shown were used as primers in the polymerase chain reactions for mutagenizing the NR cDNA. The amino acid that was mutagenized is indicated above each oligonucleotide sequence, and the corresponding codon is underlined. The second oligonucleotide used for priming the reverse strand for each mutation was the exact complement of the one shown.

NR Expression System

Wild-type and mutant forms of NR cDNA were directly inserted into the EcoRI site of the *Pichia pastoris* expression vector pHILD2 (Invitrogen, San Diego, CA). The orientation of the cDNAs was checked by restriction mapping and sequencing. The expression clones were transformed into *P. pastoris* strain GS115 (Invitrogen) or SMD1168 (gift of E. Komives, University of California, San Diego) by electroporation, as described by Becker and Guarente (1991).

Growth media and conditions for selection of transformants were as described by the manufacturer (Invitrogen). His+ transformants were selected. Cells expressing NR protein were identified as follows. To induce NR gene expression, which was driven by the alcohol oxi-dase promoter of *P. pastoris*, transformants were first grown in MGY medium (1.34% yeast nitrogen base with ammonium sulfate, 1% glycerol, and 4 x 10⁻⁵% biotin) to an OD_{600} of ~2. Cells were washed and then resuspended in MM medium (1.35% yeast nitrogen base with ammonium sulfate, 0.5% methanol, and 4 x 10⁻⁵% biotin) at the same density and grown for another 24 hr. They were collected and immediately stored at −80°C. Frozen cells were thawed on ice, and an approximately equal volume of glass beads (450 to 600 μm; Sigma)

and NR extraction buffer containing 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 0.1% Triton X-100, and proteinase inhibitors (0.4 μg/mL leupeptin and 1 μg/mL pepstatin) were added. The mixture was vortexed for 5 min and then centrifuged at 4°C for 10 min to collect the supernatant. The protein was either used directly for NR assays or desalted for the in vitro inactivation assay. Desalting of the extract was performed using a Sephadex G-25 PD-10 column, (Pharmacia) equilibrated in extraction buffer and taking the material in the void volume. NR assay was performed in a 1-mL volume containing 50 mM Mops-NaOH, pH 7.5, 5 mM $MgCl_2$, 10 mM KNO_3, 1 mM NADH for 15 min at 30°C as described by Huber et al. (1992). One unit of NR activity is defined as 1 μmol of nitrite per min at 30°C.

In Vitro Inactivation Assay

The double NR mutant of Arabidopsis, G'4-3 (Wilkinson and Crawford, 1993), was the source of plant extract for the in vitro inactivation assay. The mutant was grown in soil under constant light at 25°C for 3 to 4 weeks, and the leaf material was harvested after 15 min of dark treatment. Protein extract was prepared by homogenizing tissue in a glass homogenizer in NR extraction buffer (1 g of leaf material per mL of buffer). Homogenized material was centrifuged for 5 min at 4°C, and the supernatant was desalted using a Sephadex G-25 PD-10 column, as described above. The inactivation assay was performed first by mixing G'4-3 extract with desalted NR protein produced in *P. pastoris*. The mixture was then incubated at room temperature (22°C) for 20 min in the presence or the absence of 5 mM ATP followed by the NR assay. In the EDTA treatment, EDTA was added to a concentration of 10 mM at the beginning of NR assay.

nit-1 MoCo Complementation Assay

The *Neurospora crassa nit-1* mutant was obtained from S. Brody (University of California, San Diego). Preparation of *nit-1* extract and conditions for the molybdenum-pterin cofactor (MoCo) complementation assay were as described by Mendel et al. (1985) and LaBrie et al. (1992). One unit of MoCo specific activity is defined as restoring NR activity to 1 μmol of nitrite per min per mg of protein from the MoCo source.

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